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This award is a Predoctoral Fellowship to support the doctoral training of Donna Egender. The goal of this research is to investigate the effects of the small stress protein, hsp27, on growth and motility characteristics of normal and tumor derived human mammary cell lines. Our study is based on the hypothesis that hsp27 is a component of a signal transduction pathway that regulates actin microfilament dynamics, and may affect cell migration and the metastatic potential of tumors. We hypothesize that cells overexpessing hsp27 will show increased motility and altered chemotactic properties, in addition to increased resistance to heat killing and certain drugs. Academically, Donna has finished her formal course requirements, and successfully completed the written and oral Doctoral Preliminary Exam. Research completed this year includes preparation of constitutive and inducible expression vectors for overexpressing human hsp27 and mouse hsp25 in transfected human mammary cells. An antisense construction for downregulation of human hsp27 expression has also been prepared. Preliminary experiments to optimize transfection of human MCF7 and MDA231 cells have been performed, as well as experiments to gain facility in determining clonal survival of stress.

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INTRODUCTION

This award is a Predoctoral Fellowship to support the doctoral training of Donna Egender. This year's progress includes academic and research accomplishments.

The goal of this research is to investigate the effects of the small stress protein, hsp27, on growth and motility characteristics of normal and tumor derived human mammary cell lines. Preliminary clinical studies indicate that elevated levels of hsp27 in breast tumor cells correlates with aggressive metastasis and poor prognosis (1,13). We have shown that hsp27 overexpression confers resistance to killing by hyperthermia and by certain anti-tumor drugs (5,6). Phosphorylation of hsp27 increases rapidly in cells treated with heat, cytokines or mitogens (7,10,12,15). In rodent cells overexpressing human hsp27, the actin cytoskeleton is resistant to damage caused by hyperthermia or cytochalasin D treatment (8,9). High levels of hsp27 also correlate with increased accumulation of cortical actin and pinocytotic activity, suggesting a possible effect on cellular motility. In contrast, cells expressing a non-phosphorylatable form of hsp27 show inhibition of processes depending on cortical microfilament dynamics (9).

Our study is based on the hypothesis that hsp27 is a component of a signal transduction pathway that regulates actin microfilament dynamics, and may affect cell migration and the metastatic potential of tumors. We hypothesize that cells overexpressing hsp27 will show increased motility and altered chemotactic properties, in addition to increased resistance to heat killing and certain drugs. Overexpressing cells may respond more vigorously to chemotactic agents, or may respond to different signalling molecules than the parent cell type. We predict that the cells expressing antisense hsp27 sequences, or those expressing the unphosphorylatable mutant will show responses antagonistic to those shown by cells overexpressing normal hsp27.

We proposed to 1) prepare human mammary cell lines expressing either increased levels of hsp27, unphosphorylatable hsp27, or antisense sequences that reduce endogenous hsp27 expression; 2) assay the rate of cell proliferation in these cell lines, compared to controls; 3) assay motility and response to cytokines of these cells using the Boyden chamber technique; and 4) study the resistance of the cell lines to hyperthermia, arsenate, cytochalasin D, and antitumor drugs. These goals would be achieved through the following approaches:

a. Plasmids will be constructed that allow expression of the hsp27 gene independently of estrogen under control of a metallotheionine promoter in human mammary cell lines. Constructions expressing normal hsp27, non-phosphorylatable mutants of hsp27, and antisense sequences for down-regulation of hsp27 expression will be prepared.

b. Hsp27 gene expression will be a) downregulated in ER+ MCF7 cells by transformation with antisense gene constructions and b) induced independently of estrogen in ER negative MDA-231 cells using a metallothioneine promoter. Cells expressing different levels of hsp27 will be compared with respect to proliferation rate, general motility, chemotactic properties, and resistance to general stress or anti-proliferative drugs.

c. The importance of hsp27 phosphorylation for the effects observed in Aim 2 will be studied by transforming MDA-231 cells with genes encoding non-phosphorylatable variants of the hsp27 gene.

PROGRESS REPORT

I. ACADEMIC: During the current year, Donna has completed her formal required structured course work. She has assembled her Dissertation Committee, which includes as members: Dr. Eileen Hickey, (chair). Drs. Lee Weber and Ellen Baker, Biology Dept., and Dr. William Courchesne, Microbiology Dept., University of Nevada Reno, and, as outside member, Dr. G. Shyamala, Dept. of Cell and Molecular Biology, University of California, Berkeley. To be admitted to candidacy, students in the Cell and Molecular Biology Program at UNR are required to prepare a grant proposal in appropriate NIH or NSF format, on a topic other than their chosen thesis research. Following acceptance of the written proposal, the student gives an oral presentation and defense of the proposal. In November, 1994, Donna completed this requirement, with a proposal entitled " A study of the effects of Bcl-2 and Bax homodimerization mutants on apoptosis in a pre-B lymphocyte cell line", and successfully completed her oral defense. In addition, she presented the results of preliminary studies on estrogen induction of hsp27 gene expression in MCF7 cells at the annual CMB Program Research Meeting.

II. RESEARCH:

The goals for the first year of the fellowship were to complete preparation of the expression constructions, and to begin making the transfected cell lines. These goals have been substantially met. In addition, Donna has investigated resistance to heat killing in newly made hamster cell lines overexpressing human hsp27, in order to be prepared to perform these assays easily when the transfected mammary cell lines are available. Figures and Figure Legends are in the Appendix section of this report.

A. Construction of hsp27 expression vectors:

1. Constructions for overexpression of hsp27. We had the following plasmids for overexpression of hsp27 at hand prior to the award:

a. Constitutive expression of human hsp27: **pSV2711**, (Figure 1A). This contains the entire transcribed sequence of the human hsp27 gene (4), from an Aat II site in the 5' leader to the 3' Hind II site 3' to the transcription termination site, under control of the SV40 early promoter.

b. Constitutive expression of mouse hsp25: **pSVM25**, (Figure 1B) This contains the mouse hsp25 cDNA (gift of Dr. M. Gaestel (2)) under control of the SV40 early promoter, and upstream of the SV40 3' transcription termination and polyadenylation signals. This plasmid will be useful for immunological differentiation of the overexpressed small heat shock protein from the endogenous human protein.

c. Inducible expression of human hsp27: **pMTO2711**, (Figure 1C) This contains the entire human hsp27 gene as in a. above, under control of the ovine metallothioneine promoter (11). This promoter allows induction of expression of genes with cadmium, at levels lower than those that initiate the stress response (8). In addition, the ovine promoter is less leaky than the mouse metallothioneine promoter we initially planned to use.

Donna has made the following constructions to complete the complement of plasmids for overexpression of the small heat shock protein:

d. Inducible expression of mouse hsp25: **pMTM25**, (Figure 1D). This contains the mouse hsp25 cDNA under control of the ovine metallothioneine promoter.

e. Constitutive expression of human hsp27: **pHβSL1S**, (Figure 1E). This construction contains the human hsp27 cDNA, obtained by PCR and verified by sequencing, extending from the proximal transcription start site to the polyadenylation site. The cDNA has been inserted with linkers into the Hind III-site of the multiple cloning site of the expression vector pHβapr1-neo (3), and sense orientation determined by restriction digest. This places the cDNA under control of the β-actin promoter, chosen for its strength and expression in all cellular backgrounds.

f. Constitutive expression of mouse hsp25: **pHβM25**, (Figure 1F). This construction contains the mouse hsp25 cDNA inserted with BamHI linkers into the BamHI site of pHβapr1-neo.

2. Antisense constructions

Since the gene encoding hsp27 is unique (4), expression can theoretically be inhibited by antisense RNA (14). The first construction Donna has made for this purpose, **pHβSL1-A**, (Figure 1G), puts the human hsp27 cDNA used in e, above, in the antisense orientation under control of the β-actin promoter (3). As a second approach, Donna is placing the hsp27 cDNA in antisense direction under control of the metallothioneine promoter, using the MTO11 plasmid used in c. and d. above.

B. Transfection of human mammary cell lines:

Preliminary experiments were performed to determine optimal transfection conditions. We are using the Lipofectin reagent (GIBCO/BRL) for transfections. We will use the plasmid pPUR (Clontech) for selection when making stable cell lines. This vector contains the S. albicans puromycin-N-acetyl transferase gene, and allows selection with puromycin. We have found this an effective and inexpensive selection system. Mammary cell lines are maintained in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal calf serum and 6ng/ml insulin.

Initial experiments to determine levels of intrinsic resistance to puromycin in MCF7 cells showed clonal survival of less than 1 in 10^6 cells in puromycin at either 2.5, 5, or 10 μg/ml (data not shown). Experiments are underway to determine the optimum puromycin concentration for selection and stable maintenance of transfectants.

The optimum concentration of the Lipofectin reagent and the optimum time of exposure to the reagent for transfection of MCF7 cells was determined in a transient transfection assay, using the luciferase expression vector pLuxF3 (gift of D. Lloyd, Pfizer). MCF7 cells were plated in 35mm plates at 2 x 10^5/ plate, and allowed to attach for 12 hours. Two micrograms of pLuxF3 plasmid were introduced into the plates with 0 - 20μl of Lipofectin reagent, as indicated in Figure 2. Cells were incubated with the Lipofectin in Opti-MEM serum-free medium (GIBCO/BRL) according to the manufacturer's directions, for either 6 or 16 hours, at which time cells were rinsed and DMEM plus 5% fetal calf serum was added for further incubation. Cells were assayed for transfection efficiency 48 hours after removal of the Lipofectin. Cells were rinsed and lysed and luciferase activity was measured in a Turner Luminometer, according to the directions in the Promega Luciferase Assay kit. Luciferase activity was highest in cells exposed to the highest level of Lipofectin tested, and following the 16 hour exposure (Figure 2). This is in contrast to results previously obtained in our laboratory with hamster fibroblasts, which do not tolerate long exposure to Lipofectin. This assay is now being repeated with MDA231 cells.

C. Assaying clonal survival of heat stress in hamster fibroblasts overexpressing human hsp27:

Ultimately the human mammary cell lines to be transfected with the constructions described above will be assayed for differences in resistance to drugs and thermal stress. As preparation for these experiments, Donna assayed heat resistance in several clonal hamster cell lines being prepared in our laboratory, that had been selected in 10μg/ml puromycin following cotransfection with the human hsp27 gene p2711(4) and pPUR. Assays compared survival of incubation at 44°C in the parent cell line 023, the vector only transfected control KS1, the previously heat selected human hsp27 transfectant line 2-2 (6), and two puromycin selected lines overexpressing human hsp27: 27-1 and 27-8. Cells under puromycin selection were removed from selective media 48 hours before plating for the survival assay. Cells were plated at 5×10^4 cells per well in duplicate wells in 24-well tissue culture plates in DMEM plus 5% fetal calf serum, and allowed to attach and begin growth for 24 hours. The plates were submerged in a 44°C water bath for 1, 2, 3, and 4 hours, and then transferred to a 37°C incubator where they were incubated in an atmosphere of 5% CO_2 without disturbance for 7 days. At this time, the plates were rinsed well with phosphate buffered saline to remove dead cells, and the surviving colonies visualized by fixation and staining with Coomassie Blue. Figure 3 shows the typical survival results following exposure to 2 and 3 hours of heat stress. All of the hsp27 overexpressing cell lines show higher heat resistance than the parent cell line and the vector control. It is noted that this procedure differs from the method of assaying clonal survival we initially proposed, in which the cells were to be trypsinized and plated at known density following the heat treatment. Results of ongoing experiments for a different project in our laboratory suggest that the results of survival assays performed without trypsinization, as done here, or with trypsinization, may give significantly different results. This may be especially relevant when comparing heat resistance of cell lines overexpressing the wild type with those expressing the non-phosphorylatable mutant hsp27. The transfected mammary cell lines will be assayed using both methods in our projected research.

CONCLUSIONS

Donna has completed her formal course work and will now focus exclusively on her research. She now has hsp27 expression constructions prepared for the experiments proposed. She is initiating transfection of mammary cell lines. In the coming year, she will select and characterize the transformant cell lines. Changes in our proposed experiments include use of the pPUR puromycin resistance vector as selective marker, and the use of the ovine metallothioneine promoter as inducible promoter, because it is less leaky than the mouse promoter we originally intended to use. In addition, we will compare clonal survival of heat and drug treatments in cells allowed to grow without trypsinization, as well as in cells trypsinized and replated following treatment.

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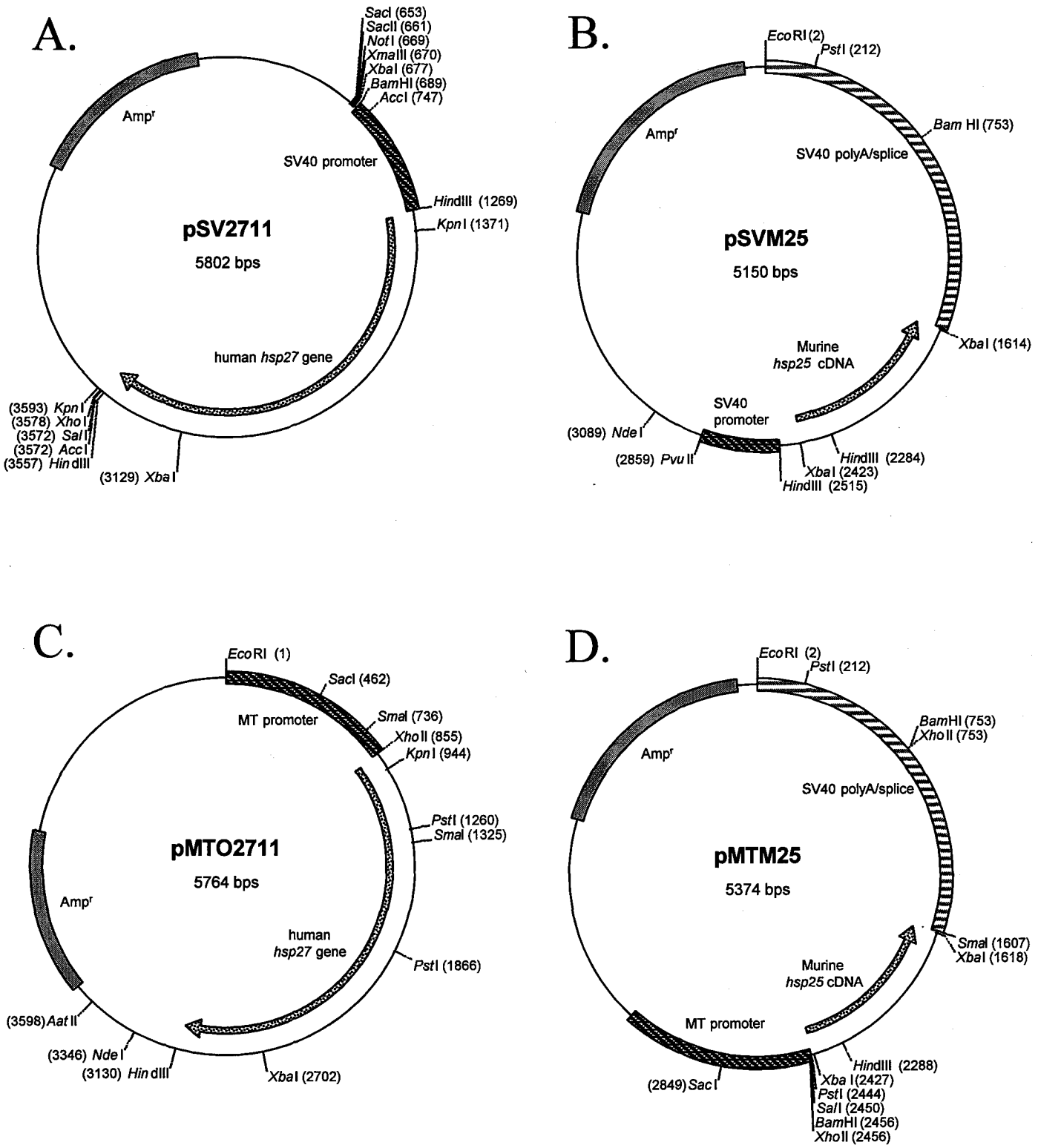
APPENDIX

Fig. 1. Diagrams of the constructions for expressing human hsp27 and mouse hsp25 in mammary cells.

Fig. 1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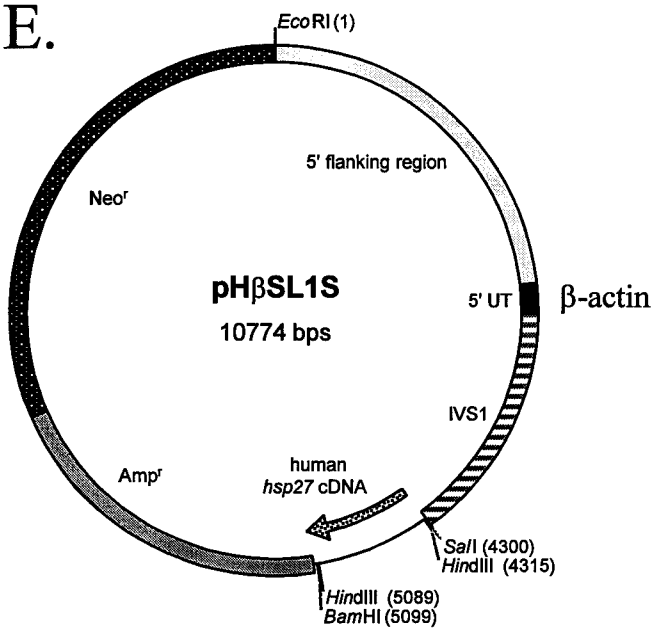
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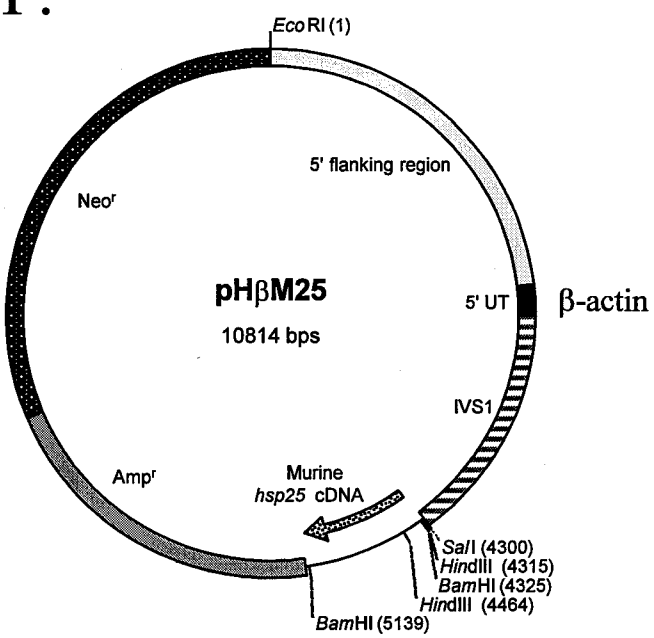
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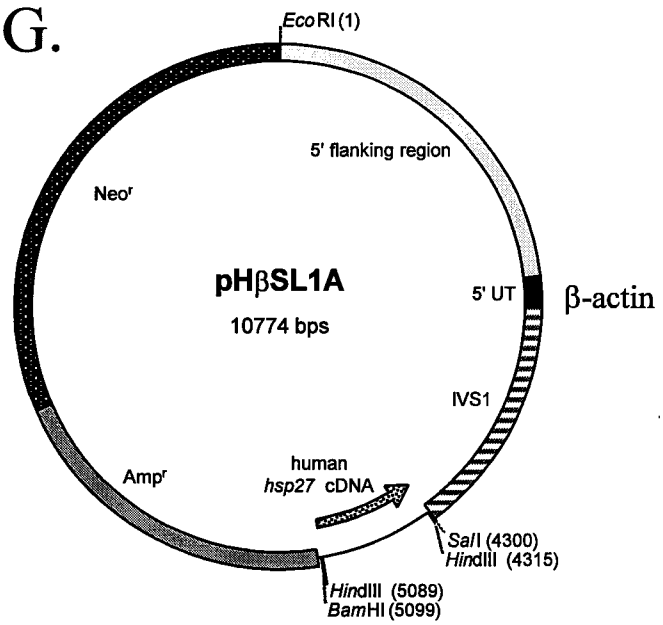
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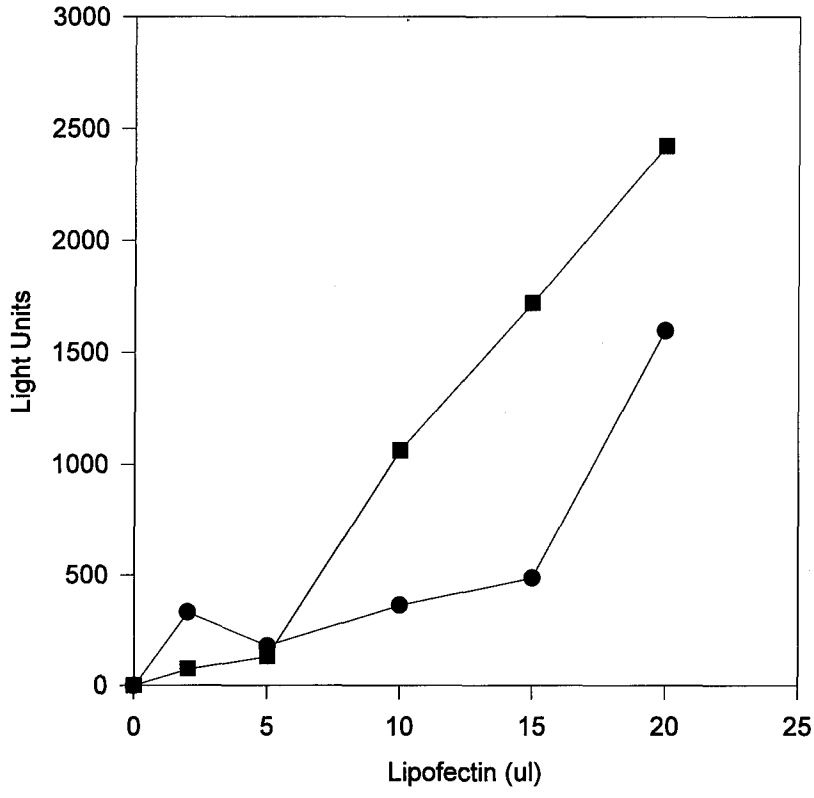

Fig. 2. Determination of optimal exposure time and amount of LIPOFECTIN reagent for transfection of MCF7 cells. Cells were plated at 2 x 10^5 cells per 35mm plate and transfected 12 hours later using 0, 2, 5, 10, 15, or 20 ul of LIPOFECTIN® reagent (Gibco-BRL) with 2 ug of plasmid pLuxF3 containing the luciferase gene under the control of the SV40 promoter. Media was replaced after 6 (●) or 16 (■) hours. Cells were rinsed and lysed 48 hours later, according to the Luciferase Assay System (Promega), and lysates stored at -70°C. A luciferase assay was performed using 10 ul of room temperature cell extract mixed with 100 ul Luciferase Assay Reagent (Promega) and luminescence was measured for 10 sec using a Model 20e luminometer (Turner Designs).

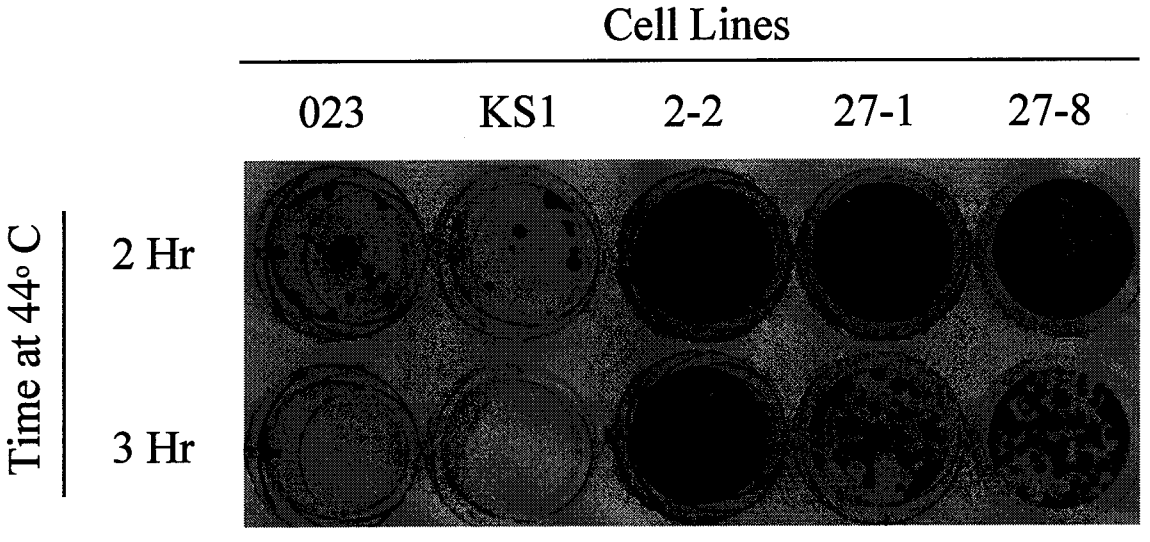

Fig. 3. Clonal survival after heat stress of stable transformed hamster fibroblasts. Cell lines were plated at 5 x 10^4 cells per well in 24-well tissue culture plates 24 hours prior to receiving a heat stress of 44°C for 1, 2, 3, or 4 hours. Plates were placed back into a 37°C incubator for one week allowing for colony formation from surviving cells. Resulting colonies were stained with 0.0625% Coomassie blue in 50% methanol and 10% acetic acid.